Altered Postreceptor Signal Transduction of Formyl-Met-Leu-Phe Receptors in Polymorphonuclear Leukocytes of Patients with Non-Insulin-Dependent Diabetes Mellitus

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The signal transduction of the formyl-Met-Leu-Phe (FMLP) receptor in polymorphonuclear leukocytes (PMNLs) from patients with non-insulin-dependent diabetes mellitus (NIDDM) was compared to that of PMNLs obtained from healthy volunteers. According to our previous studies in this group of patients neither the decrease in insulin binding capacity nor the enhanced insulin-degrading enzyme activity was involved. In control PMNLs, 10 nM FMLP induced a pertussis toxin-sensitive increase in phosphatidyl inositol (PI) cleavage and a subsequent Ca^{2+} signaling from the intracellular pools. On the other hand, the FMLP-induced protein kinase C (PKC) activation and translocation into the membrane could not be detected in these cells via the measurement of ^{32}P incorporation into histone. In contrast, in PMNLs of this special group of patients suffering from NIDDM the FMLP stimulus produced a significantly low increase in PI cleavage and Ca^{2+} signaling from the intracellular pools. Moreover, in resting PMNLs of these patients with NIDDM, not only the [Ca^{2+}]$_i$ but also the membrane-bound PKC activity was found to be significantly increased. In addition, PKC translocation into the cell membrane of diabetic PMNLs could be further triggered with FMLP as judged by the measurement of ^{32}P incorporation into histone. Based on these results, it appears that the signaling of FMLP receptors in PMNLs of some NIDDM patients may have an alternative pathway through Ca^{2+} influx from extracellular medium, arachidonic acid cascade, and PKC activation. © 1998 Academic Press

Key Words: diabetes mellitus; signal transduction; FMLP receptor; leukocyte.

INTRODUCTION

It is well known that the wide range of chemotactic products released during the immune response do not only have chemotactic effects on polymorphonuclear leukocytes (PMNLs) and macrophages, but also induce an increase in oxidative burst, an increase in arachidonic acid (AA) cascade, and an increase in the intracellular killing capability of cells (1–3). Synthetic formyl peptide formyl-Met–Leu–Phe (FMLP) is also frequently used in different studies (4, 5).

In the light of Berridge's work, our knowledge concerning the signal transduction of FMLP receptors is almost complete (6–9). It was demonstrated by many authors that human PMNLs exhibit a FMLP receptor-mediated signal transduction via a G protein, phospholipase C (PLC) activation, inositol phosphate (IP) generation, Ca^{2+} signaling, AA cascade, and a PKC activation (10–12). In addition, we have previously demonstrated that PMNLs obtained from a group of patients with (NIDDM) non-insulin-dependent diabetes mellitus carry a reduced capacity in superoxide anion generation when Fc or FMLP receptors are stimulated, whereas the intracellular killing capability of PMNLs remains unaltered (13, 14).

In our previous studies, the functions of PMNLs in healthy aged subjects were also investigated. When such PMNLs were stimulated with FMLP, the moderated alterations in effector functions (intracellular killing capability, superoxide anion generation) were associated with the disturbed signal transduction of FMLP receptors (15–18). In fact, the FMLP-stimulated PMNLs from 65- to 80-year-old subjects showed an attenuated IP$_3$ response and Ca^{2+} signaling (17, 18). It should be pointed out that the primary damage in the immune system and the PMNL-dependent susceptibility to infections in NIDDM patients receiving an adequate therapy for diabetes have not been demonstrated so far. Some disturbances in PMNL functions, due to pathologic glucose metabolism resulting in decreased glycolysis and ATP depletion, have been previously shown (19–22). However, certain pathologic alterations, as detected by an increased chemiluminescence of resting PMNLs, have also been demonstrated in NIDDM patients with normal blood glucose levels (23).

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In the present study the FMLP receptor-mediated signal transduction pathways of PMNLs obtained from a carefully selected group of patients with NIDDM were compared to control neutrophils. We demonstrate here that age-matched control PMNLs from healthy volunteers exhibit a FMLP receptor-mediated signal transduction through the well-known pathway. In contrast, in PMNLs of some patients with NIDDM an altered signal transduction of FMLP receptors was observed.

MATERIAL AND METHODS

Patients

The investigations were carried out on 38 male NIDDM patients with normal weight. The mean age was 61 ± 2.2 years and BMI, 25.5 ± 2.7 kg/m$_2$. Their disease period ranged from 5 to 11 years (mean: 8.3 ± 1.1). All underwent a glucose tolerance test to confirm a diabetic blood glucose curve. The fasting blood sugar levels were 6–12 mmol/L, while the corresponding insulin levels were above 36–38 mU/L. The patients did not receive any antidiabetic drug treatment. The mean HbAlc level was $8.2 \pm 0.8\%$. All patients received a standard diet consisting of 50% carbohydrate, 30% fat, and 20% protein per day. The daily total carbohydrate intake was 160 g. The above caloric intake was achieved in the form of five meals per day. Meal times were at 8, 10, 12, 17, and 21 hours. Case history, physical examination, and ECG revealed no detectable signs of ischemic heart disease, obliterative arteriosclerosis, and significant cerebrosclerosis. Patients' serum cholesterol levels ranged between 6.1 and 10.3 mmol/L. Those patients who showed hypophyseal or adrenal gland disorders were excluded. The control group consisted of 15 men (mean age: 65.1 ± 4.5 years) who, based on the physical, radiological, and laboratory examinations, proved to be healthy. These patients received the same standard diet as described for the NIDDM patients. To clarify the cause of insulin resistance, receptor assays were performed on PMNLs of 38 patients.

Determination of insulin receptors in PMNLs. The method described by Bar *et al.* (24) on human monocytes was used. Briefly, human recombinant ^{125}I-insulin at tyrosine A14 (Amersham, 72 TBq/mol) and unlabeled human insulin (Novo) was applied to cells in the presence of N-ethylmaleimide (NEM) to inhibit the SH-sensitive insulin-degrading enzyme (IDE). The labeled insulin was used at a concentration of 0.2 ng/ml. The PMNL suspension containing 2×10^6 cells in 1.0 ml RPMI 1640 was incubated at 15°C in the presence of 15 mM NEM for 90 min. After a vacuum-assisted washing through a Whatman G/FA, the filters were dried and

their radioactivities measured in a Packard 2200 CA scintillation counter using toluol as a solvent. The specific binding was subtracted from the total binding and the specific binding of insulin was expressed as the percentage of total activity of labeled insulin in the presence of 0.1 μg/ml cold insulin in the mixture. In the healthy control group the average in 15 men was $7.67 \pm 3.3\%$.

Determination of IDE activity in PMNLs. This method was carried out according to Theiss *et al.* (25). Briefly, 0.2 ng/ml ^{125}I-insulin (Amersham, 72 TBq/mol) and 100 ng unlabeled insulin (Novo) were added to 2×10^6 PMNLs in RPMI 1640 containing 5 mM EGTA and 1 μM reduced glutathione (Serva). After a 5-min incubation at 37°C, the reaction was terminated by adding ice-cold trichloroacetic acid (TCA). Following centrifugation, the radioactivity of the TCA-soluble fraction was measured. In control tubes the assay was performed as above but in the presence of 15 nM NEM which inhibits SH-sensitive insulin degradation by PMNLs. The IDE activity was calculated as the percentage loss of insulin during the 5-min interval from the total amounts of hormone applied. The average of IDE activity in PMNLs of 15 healthy men was $22.8 \pm 1.9\%$ SEM.

Based on the decreased ^{125}I-insulin binding capacities and increased IDE activities, 10 of 38 patients were excluded from further investigations, because the differences were higher than twice the SE obtained from the average of the control. Thus, the complete experiment was carried out on 28 patients with NIDDM, who had normal ^{125}I-insulin binding capacity and IDE activity. Venous blood samples (15–20 ml) were taken at intervals of 10–15 days from 6–8 patients and 3–4 control individuals for each set of experiments. The interassay coefficient did not exceed 20%.

Isolation of PMNLs

PMNLs were separated by Ficoll–Hypaque density gradient centrifugation according to the method of Boyum (26). The cell suspensions were 95% pure for PMNLs as judged by morphological criteria, and 96% were viable.

Culture Conditions

Cell suspensions were performed in serum-free Hanks' balanced salt solution (HBSS) with the appropriate cell densities. All incubations were carried out in a CO$_2$ incubator (CO$_2$:5%, air:95%, humidity:95%) at 37°C. The PMNLs were stimulated with 10 nM N-formyl-Met-Leu-Phe (Serva).

Measurement of Inositol Phosphates

The determinations were carried out according to the method of Dillon *et al.* (10) modified by Patthy *et al.*
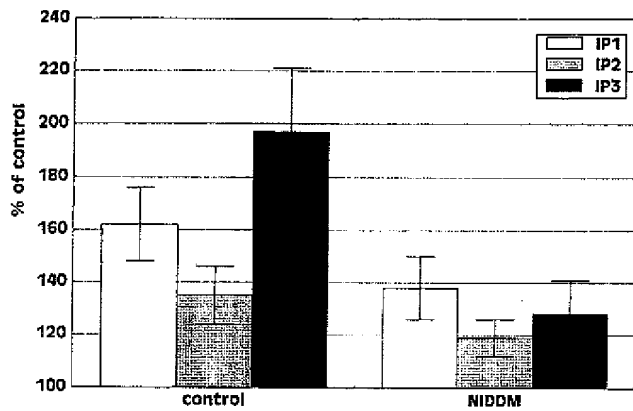
FIG. 1. The effect of 10 nM FMLP on the inositol phosphate production in PMNLs obtained from control subjects and patients with NIDDM. Each value represents the mean ± SEM of eight individuals in the control group and seven patients in the NIDDM group. The decrease of IP_1, IP_2, and IP_3 production in the NIDDM group was statistically significant ($P < 0.01$).

(27). An aliquot of PMNL suspension (10^7 cells/ml) in Hepes-buffered HBSS was preincubated for 4 hr at 37°C in the presence of 25 μM myo-[^{3}H]inositol (Amersham) and 10 mM LiCl$_3$ using a CO$_2$ incubator equipped with a shaker. Following vigorous washings, the cell-bound radioactivity was determined. The myo-[^{3}H]inositol incorporated was at least 50% of the total applied radioactivity. These cells were then stimulated with 10 nM FMLP for 20 s. Reaction was terminated with ice-cold perchloric acid and neutralized with saturated KHCO$_3$. The precipitate was centrifuged and filtered through a 0.45-μm Milipore filter. The IPs were isolated by reverse-phase ion-pair chromatography (28) using inositol monophosphate (IP_1), inositol diphosphate (IP_2), and inositol trisphosphate (IP_3) as internal standards (Amersham). Following fractionation, the radioactivities were determined in a Packard 2200 CA liquid scintillation counter. The amounts of IP_1, IP_2,

and IP_3 produced were expressed as the percentages of dpm to the corresponding resting PMNLs.

Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was determined as described by McCormach and Cobbold (29). Briefly, 1 ml PMNL suspension containing 5 × 10^6 cells plus 20 μl Indo 1/AM (Calbiochem) was incubated for 30 min at 37°C in a shaker. The cells were then washed vigorously and aliquots were resuspended in HBSS. The determination of $[Ca^{2+}]_i$ was carried out in a spectrofluorometer (Hitachi F-4500) at 405 and 485 nm under constant stirrings at 37°C. The final mixture consisting of 10^6 PMNLs in 2.0 ml HBSS was placed into a cuvette, and the cells were stimulated with 10 nM FMLP during the measurement. Preincubation was made in 7.5 nmol/ml pertussis toxin (PT, Calbiochem) for 120 min. In some experiments PMNLs were stimulated in HBSS consisting of 3 mM EGTA. The $[Ca^{2+}]_i$ levels were calculated according to the given equation (29).

Release of $[^{14}C]AA$ Metabolites

AA metabolites were determined according to the method of Boraschi et al. (30) using [^{14}C]arachidonic acid from Amersham (58.4 mCi/mmol). Cell suspensions containing 5 × 10^5 PMNLs were incubated with 0.2 μCi [^{14}C]AA for 20 min at 37°C. After vigorous washings the cell-bound radioactivity was considered the total activity. PMNLs were stimulated with 10 nM FMLP (Serva) and 1.0 μM A$_{23187}$ (Calbiochem). Following a rapid centrifugation the radioactivities of the supernatants were measured in a Packard 2200 CA scintillation counter. The percentage of the released radioactivity was calculated as the proportion of the total cell-bound activity.

The Measurement of PKC Activity

This method was carried out as described by Bell et al. (31) and modified by Gopalakrishna et al. (32). After

TABLE 1

The Cytosolic Free Ca^{2+} Level and the Ca^{2+} Signal and Its Time Dependency in FMLP-Stimulated
PMNLs of Controls and Patients with NIDDM

Exp. groups	No.	Treatment	$[Ca^{2+}]_i$ nmol/liter	Ca^{2+} signal ΔCa^{2+} nmol/liter	Time to return (seconds)
Control	12	HBSS	161.5 ± 14	97.4 ± 10	148.3 ± 16
	8	Pertussis toxin	159.2 ± 14	28.5 ± 3**	114.5 ± 10
	10	Ca^{2+} free medium	147.1 ± 15	85.4 ± 10	161.2 ± 18
NIDDM	10	HBSS	290.7 ± 24*	31.2 ± 2*	382.1 ± 41*
	7	Pertussis toxin	287.9 ± 31	35.6 ± 4	376.6 ± 32
	7	Ca^{2+} free medium	264.2 ± 30	11.1 ± 1**	241.5 ± 18

* Values differ significantly from control values ($P < 0.01$).
** The inhibition is statistically significant ($P < 0.01$).

TABLE 2

The Release of [^{14}C]AA Derivatives from PMNLs of
Controls and Patients with NIDDM

Exp. groups	No.	Stimulus	[^{14}C]AA release (% of total)
Control	14	None	9.1 ± 0.7
	12	FMLP	12.7 ± 1.4
	8	A$_{23187}$	54.5 ± 6.8
NIDDM	9	None	15.7 ± 1.1
	10	FMLP	36.2 ± 4.2*
	7	A$_{23187}$	58.9 ± 6.1

* Value differs significantly ($P < 0.01$) from value of FMLP-stimulated control PMNLs.

a 2-min stimulation with 10 nM FMLP, the PMNL suspensions (5 × 10^6 cells) were rapidly centrifuged at 4°C. The pellet was resuspended in Hepes-buffered ice-cold HBSS containing EDTA, 0.5 mmol/L EGTA, phenylmethylsulfonylfluoride (Sigma), and leupeptin (Sigma). Cells were disrupted ultrasonically (Branson Sonifier 450) and centrifuged at 100,000g for 45 min at 4°C (Beckman L5-65B). Both the supernatants containing the cytosol and the pellets were then solubilized with Chaps (Sigma) and 1% Nonidet P-40 (Sigma). The pellets were rehomogenized and centrifuged again as above. The PKC activities of cytosolic and membrane fractions were determined by measuring the ^{32}P incorporation from [^{32}P]ATP (Institute of Radiochemical Research, Budapest) into 100 μg/ml histone III-S (Sigma) in the presence of 10 mmol/L MgCl$_2$, 1.5 mmol/L CaCl$_2$, 96 μg/ml L-phosphatidyl-L-serine (Sigma), 6.5 μg/ml 1-oleoyl-2-acetyl-sn-glycerol (Sigma), 50 μmol/L adenosine 5-triphosphate Na$_2$ (ATP, Sigma), and 100–200 cpm/mg [^{32}P]ATP. The reaction was terminated after 10 min by adding ice-cold trichloroacetic chloride and bovine serum albumin as the carrier. The precipitate was then filtered through a 0.45-μm Millipore HA filter and washed in 5× 2 ml ice-cold TCA. The radioactivity was determined with a Packard 2200 CA liquid scintillation counter by using a toluol cocktail to dissolve the filters. The PKC activity was expressed as incorporated ^{32}P (pmol/min/mg protein).

Statistics

The statistical significance of the results was calculated by Student's t test for paired samples.

EXPERIMENTAL RESULTS

In the first series of experiments the effects of FMLP-induced cleavage of PI were examined in PMNLs (Fig. 1). As compared to healthy control subjects, PMNLs

from patients with NIDDM exhibited a significant decrease in the amounts of IP$_1$, IP$_2$, and IP$_3$ generated. Table 1 demonstrates that resting PMNLs from NIDDM patients have nearly twice as much [Ca^{2+}]$_i$ as resting PMNLs obtained from the control group. The FMLP-induced Ca^{2+} signaling is decreased significantly and the time of normalization appears to be prolonged in PMNLs obtained from the NIDDM group. The pretreatment with PT in control PMNLs abrogated the Ca^{2+} signaling and the peak magnitude was independent of the presence or absence of extracellular Ca^{2+}. PMNLs from the NIDDM patients showed a decrease in Ca^{2+} signaling which could not be inhibited by PT. Moreover, the Ca^{2+} signaling did not occur in a Ca^{2+}-free medium.

In the next series of experiments, the PMNLs of control and NIDDM patients were preloaded with [^{14}C]AA, and the release of [^{14}C]AA metabolites was then measured following stimulations with FMLP or A$_{23187}$ (Table 2). The control PMNLs exhibited a minimal [^{14}C]AA release following stimulation with FMLP, while the A$_{23187}$-induced release was 54.5% of the total activity as compared to the release from diabetic cells. In PMNLs of NIDDM the spontaneous release of [^{14}C]AA metabolites was slightly increased whereas the FMLP-induced [^{14}C]AA metabolite release was significantly greater (36.2%) than in the control group.

The PKC activity in resting PMNLs was also studied (Table 3). In PMNLs from patients with NIDDM the membrane-bound PKC activity and consequently the total PKC activity both increased significantly compared to the values of control PMNLs. The membrane-bound PKC activities following FMLP stimulation in control and diabetic PMNLs were also examined. As shown in Fig. 2, no changes were found in the membrane fraction of the control group during a 60-min incubation. In cells of NIDDM patients at the 5th minute following FMLP stimulation, a peak was detected in the membrane fraction followed by a long-lasting second peak with a plateau between the 25th and the 60th minutes. These results suggest a disturbed PKC

TABLE 3

Proteinkinase C Activity in Resting PMNLs Obtained from
Controls and Patients with NIDDM

Exp. groups	No.	Cytosolic fraction	Membrane fraction	Total
		pmol ^{32}P/min/mg protein		
Control	14	1945 ± 134	187 ± 21	2045 ± 186
NIDDM	11	2050 ± 186	1733 ± 158*	3907 ± 290*

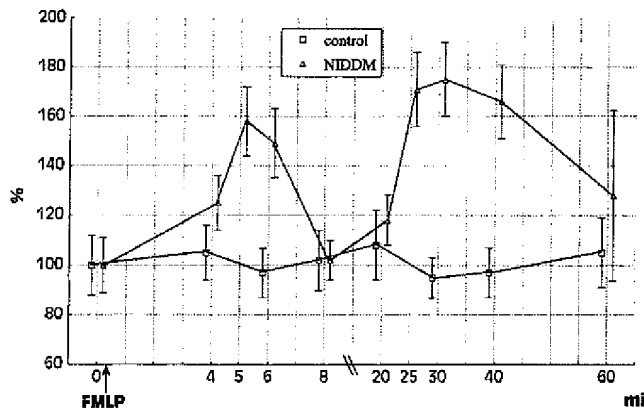* Values differ from the control value singnificantly ($P < 0.01$).

FIG. 2. The time-dependent alterations of membrane-bound PKC activity in FMLP-stimulated PMNLs obtained from controls and patients with NIDDM. Each point represents the cumulative mean ± SEM of five separate experiments carried out in 3 consecutive weeks. In these experiments, 12 control and 14 diabetic patients were included. Data are expressed as the percentages of PKC activities before the FMLP stimulation (control: 214 ± 32; NIDDM: 1598 ± 161 ^{32}P (pmol/min/mg protein)).

activation and translocation into the cell membrane in PMNLs with NIDDM when FMLP is used.

DISCUSSION

In the present study we found that FMLP-induced IP_1, IP_2, and IP_3 production and Ca^{2+} signaling were both decreased in the PMNLs of diabetic patients whereas the release of [^{14}C]AA metabolites and the PKC activation appeared to be enhanced. The experiments were carried out on PMNLs without decreased insulin receptor and increased IDE activity of 28 NIDDM patients. Subgroups were formed according to the experimental time and parameters investigated. Based on these results, it cannot be concluded that the increased PLA_2 activity is responsible for the enhanced AA cascade. Also, it is uncertain that, as an effectively functioning alternative pathway, this could inhibit the PI cleavage following a FMLP stimulation (33). However, the high $[Ca^{2+}]_i$ level in resting PMNLs from diabetic patients may play a role in the activation of PLA_2 (34). The increased $[Ca^{2+}]_i$ in resting diabetic PMNLs could either be explained by the elevated number or altered functional activity of the voltage-dependent Ca^{2+} channels, or by the decreased number and/or function of CaM-dependent Ca^{2+}-ATPase in the membrane (35). The decreased amounts of CaM in cells of diabetic patients have been described by many authors (36–39). The PMNLs and monocytes from elderly individuals also have an increased $[Ca^{2+}]_i$ and disturbances in their Ca^{2+} transport through the membrane-bound Ca^{2+}-ATPase can also be demonstrated (40).

The increased activity of membrane-bound total PKC in resting PMNLs from diabetic patients, in association with an increase in $[Ca^{2+}]_i$, may be a parameter that reflects damaged signal transduction in diabetic PMNLs. In addition, the FMLP-elicited PKC translocation into the membrane was obvious only in diabetic cells, but not in control neutrophils. As demonstrated, the curve describing the time-dependent PKC translocation had an early peak followed by a second, long-lasting peak. In this respect, it has also been reported that PKC activation and translocation by FMLP could be detected in normal human PMNLs only after a cytochalasin B treatment or when a labeled phorbol ester, [^{3}H]PDBu, was used to determine the PKC translocation (41).

These results suggest that PKC may play a role in the altered signal transduction of FMLP-stimulated diabetic PMNLs. One of the known effects of PKC is the phosphorylation of the PT-sensitive G protein at serine sites which results in a receptor uncoupling from PLC (42). This could be one of the explanations for the altered signal transduction of the FMLP receptor in PMNLs from patients with NIDDM. On the other hand, the G protein functions have been proved to be altered in diabetes (43, 44).

In conclusion, it is certain that PMNLs of patients with NIDDM have a decreased FMLP-induced superoxide anion generation (13, 14). The induction of this decreased respiratory burst is known to occur via an altered signal transduction pathway. The FMLP-induced intracellular alterations are characterized by: (i) a decreased IP_3 production, (ii) an increased $[Ca^{2+}]_i$ level and a decreased Ca^{2+} signaling, (iii) an increased AA cascade, and (iv) an increased and altered PKC translocation into the membrane. The above alterations in the FMLP receptor signaling in PMNLs from patients with NIDDM make it probable that the PLC–IP_3–Ca^{2+}–PKC pathway in the other cells and/or other receptors may also be altered.

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